JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

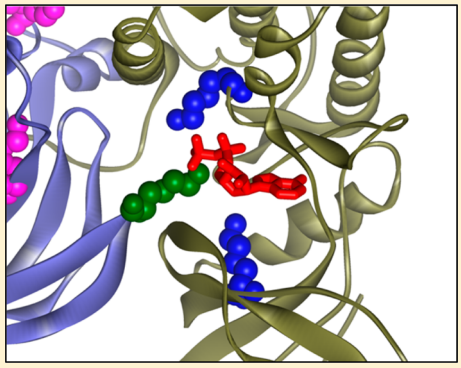
Monitoring Native p38α:MK2/3 Complexes via Trans Delivery of an ATP Acyl Phosphate Probe

Eric S. Okerberg, Heidi E. Brown, Lauro Minimo, Senait Alemayehu, Jonathan Rosenblum, Matt Patricelli,[†] Tyzoon Nomanbhoy, and John W. Kozarich*

ActivX Biosciences, Inc., 11025 North Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: Here we describe a chemical proteomics strategy using ATP acyl phosphates to measure the formation of a protein:protein complex between p38α and mapkap kinases 2 and/or 3. Formation of the protein:protein complex results in a new probe labeling site on p38α that can be used to quantify the extent of interaction in cell lysates and the equilibrium binding constant for the interaction in vitro. We demonstrate through RNA interference that the labeling site is dependent on formation of the protein:protein complex in cells. Further, we identify that active-site-directed, small-molecule inhibitors of MK2/3 selectively inhibit the heterodimer-dependent probe labeling, whereas p38α inhibitors do not. These findings afford a new method to evaluate p38α and MK2/3 inhibitors within native biological systems and a new tool for improved understanding of p38α signaling pathways.



INTRODUCTION

Activity-based probes (ABPs) are valuable tools for interrogating enzymatic activities in unpurified biological samples. Numerous ABPs have been devised, including selective reagents for serine hydrolases, caspases, cathepsins, and protein kinases.[1] These chemical tools provide a means to globally profile enzymatic activity and/or protein content in response to biological stimuli or inhibitor treatment across an enzyme family in both cell and tissue lysates.

An increasingly important application of ABPs is inhibitor selectivity profiling of endogenous protein kinases using nucleotide acyl phosphate probes (Figure 1a). These probes covalently modify conserved lysine residues in the ATP binding site of kinases and other ATPases, providing the means to measure both inhibitor affinity and selectivity across a broad range of related enzymes.[2,3] Unexpected labeling events outside the ATP-binding pocket have been observed, and the possibility that they represent previously undefined nucleotide binding sites or result from trans-protein delivery of probe during protein:protein interactions has been the subject of much speculation. Here we report the direct observation of a protein:protein interaction using an active-site-directed chemical probe for the first time. Identification and quantitation of the p38α:mapkap kinase 2 and/or 3 (MK2 and/or MK3) complexes in biological extracts illuminates this interesting property of nucleotide acyl phosphate probes.

The p38 mitogen-activated protein kinase alpha (p38α) is of particular interest as an essential component of the inflammatory response, and modulation of p38α activity has been investigated as a potential treatment for chronic inflammatory diseases including Crohn's disease and rheumatoid arthritis.[4,5] Despite intensive effort throughout the pharmaceutical industry, safe and effective p38α inhibitors have not yet been approved for clinical use. Alternatively,

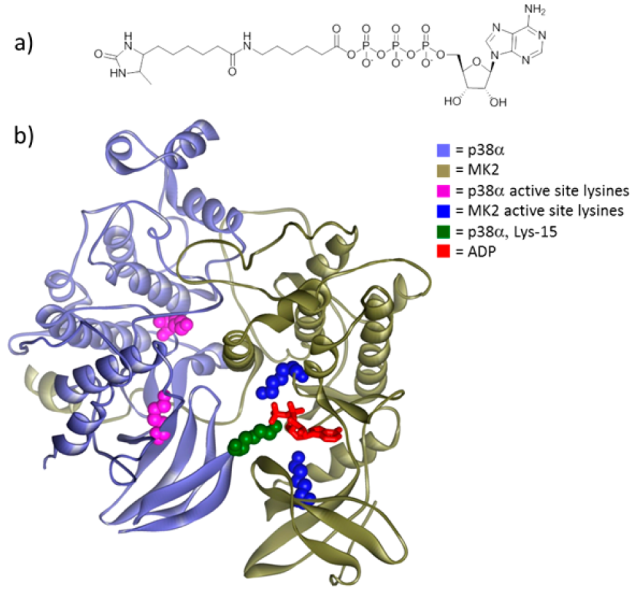


Figure 1. (a) Structure of a representative ATP acyl phosphate chemical probe used in these studies.[3] (b) Crystal structure of the p38α:MK2 complex manually overlaid with ADP from the crystal structure of ADP-bound MK2 (PDB IDs: 2OZA, 1NY3).[6−8] Lys15 of p38α is highlighted in green.

Received: December 20, 2013
Published: March 6, 2014

   dx.doi.org/10.1021/ja4129907 | J. Am. Chem. Soc. 2014, 136, 4664−4669

several groups have attempted to target pathways downstream of p38α. One branch of the p38α signaling pathway involves activation of p38α, leading to its transport into the nucleus, where it forms a heterodimer with one of its substrates, mitogen-activated protein kinase-activated kinase-2 (MK2). Formation of this tight complex (K_d = 2.5 nM) is driven by the high affinity of MK2's C-terminal tail for a docking groove on p38α.[6] Phosphorylation of MK2 at Thr-334 exposes a nuclear export signal, leading to translocation of the p38α:MK2 complex into the cytoplasm, where MK2 acts on downstream targets such as heat-shock protein 27 (HSP27) and tristetraprolin (TTP).[7,8] MK3 is a close homologue to MK2 and is believed to provide redundant/overlapping function in the inflammatory response.[7,8] The capacity to monitor the endogenous p38α:MK2/3 complex with an active-site-directed probe may further elucidate the complex signaling pathways mediated by p38α and afford a new tool for the identification of kinase/substrate pairs.

RESULTS AND DISCUSSION

The ATP acyl phosphate probe employed in this study has been shown to preferentially label protein kinases on conserved lysines within the active site that are adjacent to the ATP-binding cleft.[2,3] Labeling of these lysines is inhibited by active-site-directed inhibitors, enabling determination of inhibitor selectivity for essentially any kinase. We have consistently observed a strong signal for an unanticipated probe-labeling site on p38α Lys-15, which is distal to the ATP binding site (with ~29 Å between the epsilon amino groups on Lys-152 and Lys-15, PDB ID: 3GCP). This labeling event has been observed in numerous tissue homogenates and cell line extracts (Figure S1, Supporting Information) and led to our interest into its biological relevance. To further evaluate the nature of the p38α Lys-15 labeling in a simplified system, p38α was expressed in *E. coli*, and the resulting cell lysate was analyzed using the ATP acyl phosphate probe. Surprisingly, no Lys-15 labeling was detected (Figure 2, blue trace), even though strong labeling of an active-site lysine was observed (Lys152, two amino acids downstream from the catalytic aspartate). This implies that labeling of Lys-15 is dependent on a factor uniquely present in the mammalian protein extracts and cell lysates previously evaluated.

Heterodimer-Dependent Probe Labeling. Crystal structures of the p38α:MK2 heterodimer indicate that the p38α Lys-15 is directed into the active site of MK2 (Figure 1b), and recent experiments with deuterium exchange mass spectrometry showed that the p38α Lys-15 region has a tight interaction in the p38α:MK2 complex.[9−12] These reports prompted our further investigation into whether acyl phosphate probe labeling of p38α Lys-15 is dependent on formation of a p38α:MK2 complex. We found that, in contrast to free p38α labeling, the addition of recombinant MK2 to p38α in *E. coli* lysate resulted in strong labeling of p38α Lys-15 (Figure 2, red trace). Interestingly, no significant change was observed for the p38α active-site lysine peptide in the presence of MK2, suggesting that probe-labeling events at the two sites are independent of one another. Titration of both unactive MK2 (not activated in vitro) and active MK2 (in vitro activated) with p38α in *E. coli* lysate demonstrated a typical sigmoidal dose response for p38α Lys-15 labeling (Figure 3). Figure S2 (Supporting Information) illustrates a representative extracted ion chromatogram and the MS^2 spectrum for the probe-modified Lys-15 peptide. Binding constants measured using the

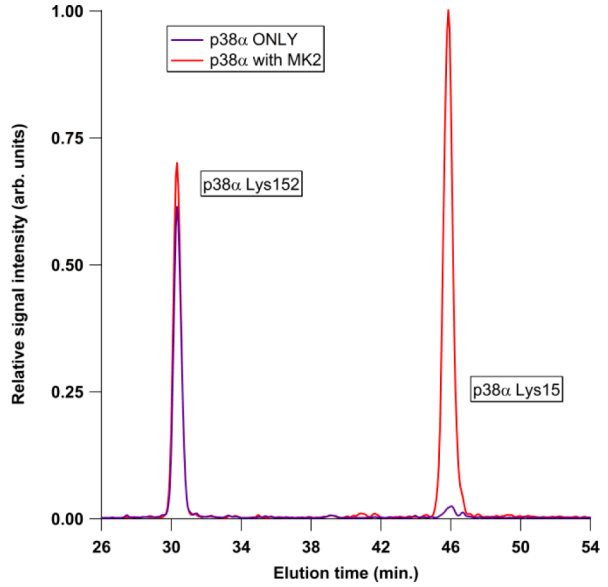


Figure 2. Heterodimer-dependent probe labeling of p38α Lys15. These extracted ion chromatograms illustrate acyl phosphate probe labeling of recombinant p38α (50 nM) in the absence and presence of recombinant MK2 (100 nM). Labeling of p38α Lys15 is dependent on heterodimer formation.

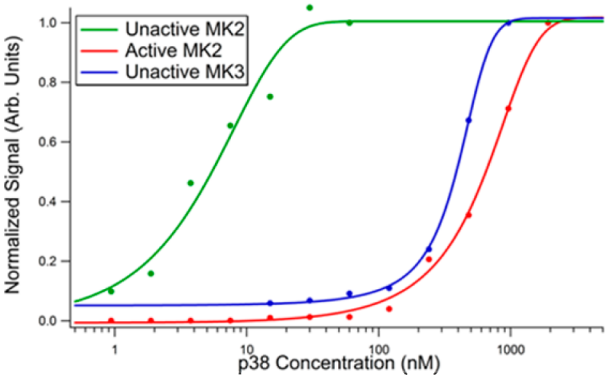


Figure 3. Titration of MK2/3 isoforms with p38α. Lysates of *E. coli* with various concentrations of p38α were incubated with 2.2 nM active MK2, unactive MK2, and unactive MK3. Calculated K_d values based on probe labeling of p38α Lys-15 are as follows: unactive MK2, 5.1 nM; active MK2, 880 nM; unactive MK3, 425 nM. Extracted ion signal from MS^2 spectra for p38α Lys-15 from duplicate analyses are plotted. Active MK3 was also evaluated under identical conditions, but the response did not reach saturation under these experimental conditions.

ATP acyl phosphate probe were K_d = 5.1 nM for unactive MK2 and K_d = 880 nM for active MK2, consistent with literature reports based on surface plasmon resonance (2.5 and 104 nM, respectively).[6] As expected, active-site labeling of p38α followed the expected linear response with increasing p38α concentration (data not shown).

Specificity of Lys-15 Labeling. Because there are several known binding partners for p38α, we investigated whether other recombinant proteins might also enable probe labeling of Lys-15. Of the proteins examined (MK2, MK3, MK5, MKK6, and TAK1), only MK2 and the closely homologous MK3 induced significant labeling of Lys-15 in the p38α-expressing *E. coli* lysate. Labeling of p38α Lys-15 was evaluated with unactive MK3, and the apparent affinity was much weaker compared to

dx.doi.org/10.1021/ja4129907 | J. Am. Chem. Soc. 2014, 136, 4664−4669

Table 1. Inhibition Profiles for SB203580 and PF3644022 in Two HL60 Cell-Based Probe Labeling Assays[a]

		Lysate Treatment				Live Cell Treatment			
		SB203580		PF3644022		SB203580		PF3644022	
Kinase	Labeling Site	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM
MAPKAPK3	Active Site, Lys173	23	6	>90	>90	12	-14	>80	63
MAPKAPK3	ATP Loop, Lys57	18	24	-180	-150	-84	-65	-320	-300
p38α	Active Site, Lys152	98	97	-6	-1	>94	80	31	35
p38α	Heterodimer Site, Lys15	44	47	98	87	-97	-20	78	61

[a]Percent inhibition values are listed for the p38α active site (Lys152), the p38α:MK2/3 interaction site (Lys15), the MK3 active site (Lys73), and an MK3 ATP loop labeling site (Lys57).

that of MK2 (425 nM for unactive MK3, Figure 3). Active MK3 was evaluated in a similar experiment, but saturation of Lys-15 was not observed over the concentration range examined (data not shown, apparent $K_d \geq 980$ nM). To our knowledge, there are no other reports that directly measure the extent of interaction between MK3 and p38α.

Profiling Small-Molecule Inhibitors of p38α and MK2/3. The crystal structure of the p38α:MK2 heterodimer suggests that p38α Lys-15 probe labeling will be sensitive to MK2/3 inhibitors, but not p38α inhibitors. To test this hypothesis, two small-molecule ATP competitive inhibitors were profiled with the ATP acyl phosphate probe, and both the active site and Lys-15 of p38α were evaluated in the context of the endogenous p38α:MK2/3 complex. The p38α inhibitor SB203580 (IC_{50} = 43 nM, Invitrogen SelectScreen) and the MK2/3 inhibitor PF3644022 (MK2, IC_{50} = 5.2 nM[13]) were selected due to their relative in vitro potency and limited off-target activity (Table S1, Supporting Information). Chemical structures are available in Figure S3 (Supporting Information).

The compounds were first profiled with the acyl phosphate probe at 10 μM and 1 μM in HL60 lysate (Table 1). Consistent with our prediction from the crystal structure, the p38α inhibitor SB203580 potently inhibited p38α active-site labeling (Lys-152) and partially inhibited Lys-15 labeling (maximal inhibition ∼45%). In contrast, the MK2/3 inhibitor PF3644022 completely inhibited both MK3 active-site labeling and p38α Lys-15 labeling, with no effect on the p38α active site. Interestingly, PF3644022 also caused an ∼2.5-fold increase in the probe labeling of the MK3 ATP loop peptide. The MK2 probe-labeled peptides did not yield sufficient signal for quantitation; complete inhibition profiles are shown in Table S1.

The compounds were next profiled in HL60 cells using a live cell format. Cells were treated overnight with the appropriate small-molecule inhibitor at 10 μM and 1 μM, and, after excess inhibitor was washed away, the cells were lysed, labeled immediately with the ATP probe, and analyzed as described previously.[2,3] While the lysate assay monitors changes in probe labeling due to the direct interaction between the inhibitor and target, the observed probe labeling profile in live cell experiments may also reflect the cellular response to inhibitor treatment. As such, overnight small-molecule incubation was performed to maximize the observed cellular response. Inhibition profiles were qualitatively similar between the live cell and lysate treatment (Table 1), but inhibition values in the live cell treatment appear slightly weaker compared to those in the lysate treatment. Compound dilution upon cell lysis, prior to the addition of probe, likely accounts for these minor differences. Interestingly, in the live cell assay, PF3644022 resulted in a >4-fold increase in probe labeling of the MK3 ATP Loop peptide. Combined, the structural and probe profiling data indicate that Lys-15 labeling is dependent upon formation of an interfacial ATP binding site between p38α and MK2/3 and that p38α probe modification occurs as a trans-protein reaction. While PF3644022 inhibition of p38α Lys-15 may be predicted from its proximity to the MK2 ATP binding site, the contribution of Lys-15 to ATP binding affinity, catalytic activity, etc. remains to be determined. The fact that SB203580 treatment does not alter p38α Lys-15 labeling in lysates indicates that these two ATP binding sites are independent, and that occluding to the p38α active site does not directly alter the orientation of Lys-15 in the heterodimer (as determined by the labeling efficiency).

One of the more striking observations from profiling PF3644022 is the strong increase observed in MK3 ATP loop labeling (2.5- to 4-fold). Surprisingly, the increase in MK3 ATP loop labeling occurs when the MK3 ATP active site is completely inhibited. This large increase may result from simple inhibitor-induced rearrangement of the MK3 active site within the heterodimer, resulting in an increase in probe labeling. Alternatively, the observed increase in probe labeling may result from some higher order biological response, including other protein−protein interactions, MK3 phosphorylation, etc. Importantly, these results demonstrate that ATP acyl phosphates afford characterization of native enzymes in ways that may be difficult to predict on the basis of recombinant enzyme assays or crystallographic information.

Heterodimer Integrity. Potent inhibition of both p38α Lys-15 and the MK3 active site, with the concomitant increase in probe labeling at the MK3 ATP loop, suggests that dramatic conformational changes in the p38α:MK3 heterodimer are induced upon PF3644022 binding, potentially leading to the complete dissociation of the heterodimer complex. To address this possibility, we investigated the interaction between PF3644022 and the p38α:MK2 complex in a homogeneous time-resolved fluorescence (HTRF) assay. We first measured the binding affinity of this interaction by titrating recombinant, unactive, polyhistidine-tagged p38α with recombinant, unactive, glutathioine-s-transferase-tagged MK2 and found the binding constant to be K_d = 0.5 nM (Figure 4). We then determined whether the presence of saturating amounts of inhibitor (10 μM) had an impact on the observed binding affinity. Neither compound altered the apparent K_d value relative to a DMSO control (Figure 4). Therefore, neither inhibitor disrupts formation of the p38α:MK2 complex, even though PF3644022 potently inhibits p38α Lys-15 labeling under similar conditions. This result may not be surprising,

dx.doi.org/10.1021/ja4129907 | J. Am. Chem. Soc. 2014, 136, 4664−4669

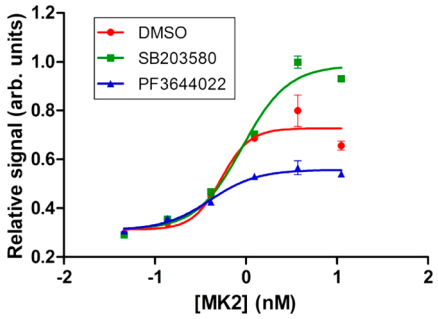


Figure 4. Homogenous time-resolved fluorescence (HTRF) signal indicating formation of the p38α:MK2 complex in the presence of saturating inhibitor. The heterodimer dissociation constant is unchanged in the presence of saturating inhibitor (10 μM), with K_d = 0.5 nM (DMSO), 0.9 nM (SB203580), and 0.4 nM (PF3644022). The altered maximal energy-transfer efficiency, however, indicates significant inhibitor-induced conformational changes.

given the fact that the heterodimer binding interaction is driven by the strong affinity of the MK2 c-terminal tail for the docking domain on p38α. However, inhibitor binding may significantly alter the heterodimer conformation, as evidenced by the altered maximal Förster resonance energy transfer efficiency in the presence of saturating levels of inhibitor (Figure 4). It is also worth noting that the p38α Lys-15 equivalent residue in p38β is also robustly labeled in lysates, while those in p38γ and p38δ are not. This is consistent with literature reports demonstrating that p38β also complexes with MK2 and MK3[14] and indicates that p38γ and p38δ may not contribute significantly to the fraction of bound MK2/3.

MK2 and MK3 Knockdown. To further explore the role of MK2 and MK3 in p38α Lys-15 labeling, we evaluated extracts of HeLa cells in which MK2 or MK3 had been depleted (>95%) by RNA interference (Figure S4, Supporting Information). Loss of p38α Lys-15 labeling was observed with MK3 knockdown as opposed to MK2 knockdown (Figure 5), suggesting that, in the resting HeLa cells, MK3 is the dominant p38α binding partner. In the MK2 knockdown samples, a modest reduction in probe labeling of the MK2/3 ATP binding pocket was observed; this peptide is redundant

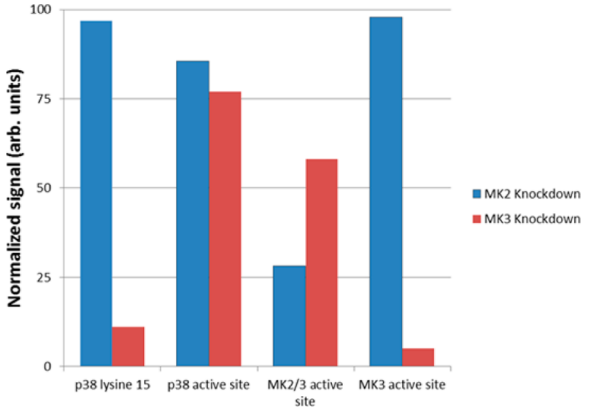


Figure 5. Signal changes in HeLa cells with MK2 and MK3 knockdowns from duplicate analyses. Signal listed as fraction of mock transfected HeLa cells. MK2 and MK3 have one labeled active-site peptide that is identical between the two, so they cannot be distinguished on the basis of the signal from that site. MK3 does have another active-site peptide that is detectable and unique to MK3.

between MK2 and MK3 and therefore confirms the presence of functional MK2 in the HeLa cells. The correlation of p38α Lys-15 labeling with MK3 rather than MK2 in HeLa cells (Figure 3) is a surprising result, given the higher affinity binding of MK2 to p38α in recombinant systems. Interestingly, a recent study has shown that MK3 acts as a negative regulator of MK2 signaling through binding to p38α under resting conditions and therefore prevents p38α:MK2 complex formation.[15] The data presented here further suggest that MK3 is the predominant in vivo binding partner for p38α in HeLa cells under standard, nonstimulated cell culture conditions and leave open the possibility that MK2 binding may be required to elicit the primary signal transduction response.

CONCLUDING REMARKS

In total, these findings illustrate unanticipated value for activity-based proteomics in profiling native protein kinases and suggest new opportunities to expand the reach of chemical proteomics. In the intensively studied p38α signaling cascade, ATP acyl phosphates not only quantify the occupancy of p38α, MK2, and MK3 active sites in the context of the p38α:MK2/MK3 heterodimer, but also enable quantitative measure of the extent of the p38α:MK2/3 interaction. This unique capability may facilitate the development of agents that functionally disrupt heterodimer formation and requisite signaling pathways within cells. For example, one may speculate that active-site-directed inhibitors blocking all p38α kinase activity may incur more dramatic biological outcomes compared to those that only disrupt the p38α:MK2/3 heterodimer. We have, in fact, profiled small-molecule inhibitors that selectively prevent p38α Lys-15 labeling (data not shown), indicating that this type of selectivity is feasible. Lys-15 labeling may, therefore, provide a new metric to evaluate p38α inhibitors and the biological outcomes of their use.

The proximity of p38α Lys-15 to the annotated Thr-16 phosphorylation site raises some interesting questions. One hypothesis is that the positive charge on Lys-15 directs Thr-16 into the MK2 active site by coordinating MK2-bound ATP. This may orient Thr-16 for a trans-phosphorylation reaction and ultimately provide a feedback mechanism within this complex signaling pathway (in contrast to the canonical MKK3/6→p38→MK2 signaling cascade). A similar mechanism for arginine-directed phosphorylation has been documented for cAMP-dependent protein kinase (PKA). Mutation of an individual arginine residue (Arg-165) adjacent to a known phosphorylation site on the PKA activation loop (Thr-197) dramatically limits auto-phosphorylation and ultimately PKA kinase activity.[16] In fact, we frequently observe probe labeling of activation loop lysine residues adjacent to annotated phosphorylation sites (data not shown), and ultimately this purported hypothesis may prove a general mechanism for kinase activation. Under this hypothesis, MK2 may actually phosphorylate p38α at Thr-16 and subsequently provide a mechanism for feedback.

The idea that chemical probes with specific recognition elements and reactive groups can undergo trans labeling events is one that has been considered recently.[17−19] While we demonstrate acyl phosphate trans labeling specifically for the p38α:MK2/3 heterodimer, the possibility exists that this is a general mechanism for acyl phosphate labeling, especially given the tremendous number of presently uncharacterized labeling events in a given sample. Moreover, the reactive groups/recognition elements may be tailored for a given enzymatic

dx.doi.org/10.1021/ja4129907 | J. Am. Chem. Soc. 2014, 136, 4664−4669

mechanism; for example, acyl phosphates of GTP have already been developed,[3] as have phosphoramidates, bifunctional probes with two reactive groups, etc.[17−19] Such probes may enable identification and quantitation of a wide range of trans labeling events as well as chemical/biological agents that disrupt them.

METHODS

MK2/3 RNAi. Chemical RNAi transfection of MK2 and MK3 was induced with 180 pmol of ValidatedStealth RNAi (Invitrogen) targeting MK2 (12936-45), MK3 (12936-46), or control (12935-200). StealthRNAi reagents were prepared in 2 mL of serum-free DMEM and added to a 10 cm culture dish. A 24 μL portion of Lipofectamine RNAiMAX (Invitrogen 13788-150) was added and mixed gently prior to room temperature incubation for 20 min. Next, 10 mL of HeLa cells in complete DMEM (9×10^5 cells) was added to the plate, mixed gently, and incubated at 37 °C until harvest (10 nM RNAi final concentration). Cells were harvested after 72 h and processed for Western blot to determine the gene silencing efficiency. Probe-labeling experiments were performed as described below on samples normalized for total protein concentration.

Bacterial Expression of p38α. First, 50 mL of LB medium containing 100 μg/mL of carbenicillin was inoculated with p38α expressing *E. coli* stock and incubated at 37 °C with shaking overnight at 250 rpm. After 16 h, these samples were diluted 100-fold into fresh media and incubated at 37 °C until the optical density at 600 nm was 0.6−0.8 unit. Protein expression was induced with 0.3 mM IPTG for 4 h at 30 °C, and cells were pelleted at 9000*g* using a J10 rotor. Pellets were resuspended with MPER reagent and centrifuged (9000*g*) to pellet the insoluble fraction. The supernatant was retained for use in various assay formats.

Analysis of Probe-Labeled Peptides. Samples were prepared for LC-MS^2 analysis as described previously.[2,3,20] Briefly, cells were lysed by sonication and gel-filtered into 20 mM HEPES, pH 7.8, 150 mM NaCl, 0.1% Triton X-100, and 20 mM $MnCl_2$. Inhibitor(s) were added for 15 min prior to initiation of the probe-labeling reaction by addition of 5 μM des-thiobiotin ADP or ATP probe. For the p38:MK2 titration experiments, purified, recombinant enzymes (MK2, Millipore 14-349; MK3, Millipore 14-586) were added to non-transfected *E. coli* lysate, which was then combined with an appropriate amount of p38α-expressing *E. coli* lysate to obtain the desired concentrations of MK2/3 and p38α and a constant total protein concentration. After probe labeling, samples were denatured and reduced (6 M urea, 10 mM DTT, 65 °C, 15 min), alkylated (40 mM iodoacetamide, 37 °C, 30 min), and gel-filtered (Bio-Rad 10DG) into 10 mM ammonium bicarbonate, 2 M urea, 5 mM methionine. Proteolytic digestion was achieved with 0.015 mg/mL trypsin (Promega PR V5113) at 37 °C for 1 h. The probe-labeled peptides were captured using 12.5 μL of streptavidin-conjugated agarose beads (Thermo, 20359) and washed. The wash cycle (150 μL/wash) included 10 washes with (A) 1% triton, 0.5% tergitol, 1 mM EDTA in PBS; 60 washes with (B) PBS; and 15 washes with (C) HPLC grade water. Probe-labeled peptides were then extracted from the streptavidin beads with two 35 μL washes of 50% acetonitrile, 0.1% trifluoroacetic acid; the resulting samples were dried and stored at −80 °C prior to LC-MS^2 analysis.

Probe-labeled peptide samples were analyzed on Thermo LTQ-Velos ion trap mass spectrometers coupled with Agilent 1100 series micro-HPLC systems as described previously.[2,3,20] Briefly, liquid chromatography was performed in 0.18 × 100 mm columns packed with 5 μm diameter, 300 Å Magic C18 stationary phase (Michrom Bioresources). Ionization at the nanospray source (Thermo Scientific) was achieved under the following conditions: 1.6 kV spray voltage, −200 °C capillary temperature, 46 V capillary voltage, 120 V tube lens voltage, 35% relative collision energy. Samples were analyzed in a "targeted" mode such that specified mass-to-charge ratios (corresponding to specific probe-labeled peptides) were specifically analyzed at the appropriate elution times.

Homogenous Time-Resolved Fluorescence (HTRF) Assays. HTRF experiments were performed with purified, recombinant polyhistidine-tagged p38α (Millipore 14-588) and GST-tagged MK2 (Millipore 14-349). HTRF assay components were purchased from CisBio, including anti-His cryptate (61HISKLA) and anti-GST D2 (61GSTDLA). Assays were performed with 2.5 nM p38α and a titration of MK2 in the presence of either 10 μM SB203580 (Millipore 559389) or 10 μM PF3644022 (Tocris 4279). HTRF signal was measured on a BioTek Synergy 2 plate reader with 330 nm excitation, 620/665 nm emission, 100 μs delay, and 300 μs collection time.

ASSOCIATED CONTENT

Supporting Information

Cell line and tissue distribution of observed p38α Lys15 labeling, representative LC-MS^2 data, selectivity profiling of SB203580 and PF3644022, and RNAi depletion of MK2 and MK3. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

johnk@activx.com

Present Address

[†]M.P.: Wellspring Biosciences, 3210 Merryfield Row, San Diego, CA 92121

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Jane Wu, Chris Herring, Arwin Aban, and Ann Shih for assistance with data collection. We thank John Kyriakis for sharing the pMT3 p38 plasmid.

REFERENCES

(1) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383.

(2) Patricelli, M. P.; Nomanbhoy, T. K.; Wu, J.; Brown, H.; Zhou, D.; Zhang, J.; Jagannathan, S.; Aban, A.; Okerberg, E.; Herring, C.; Nordin, B.; Weissig, H.; Yang, Q.; Lee, J. D.; Gray, N. S.; Kozarich, J. W. *Chem. Biol.* **2011**, *18*, 699.

(3) Patricelli, M. P.; Szardenings, A. K.; Liyanage, M.; Nomanbhoy, T. K.; Wu, M.; Weissig, H.; Aban, A.; Chun, D.; Tanner, S.; Kozarich, J. W. *Biochemistry* **2007**, *46*, 350.

(4) Schindler, J. F.; Monahan, J. B.; Smith, W. G. *J. Dental Res.* **2007**, *86*, 800.

(5) Goldstein, D. M.; Gabriel, T. *Curr. Top. Med. Chem.* **2005**, *5*, 1017.

(6) Lukas, S. M.; Kroe, R. R.; Wildeson, J.; Peet, G. W.; Frego, L.; Davidson, W.; Ingraham, R. H.; Pargellis, C. A.; Labadia, M. E.; Werneburg, B. G. *Biochemistry* **2004**, *43*, 9950.

(7) Gaestel, M. *Nat. Rev. Mol. Cell. Biol.* **2006**, *7*, 120.

(8) Ronkina, N.; Kotlyarov, A.; Dittrich-Breiholz, O.; Kracht, M.; Hitti, E.; Milarski, K.; Askew, R.; Marusic, S.; Lin, L. L.; Gaestel, M.; Telliez, J. B. *Mol. Cell. Biol.* **2007**, *27*, 170.

(9) Davidson, W.; Frego, L.; Peet, G. W.; Kroe, R. R.; Labadia, M. E.; Lukas, S. M.; Snow, R. J.; Jakes, S.; Grygon, C. A.; Pargellis, C.; Werneburg, B. G. *Biochemistry* **2004**, *43*, 11658.

(10) ter Haar, E.; Prabhakar, P.; Liu, X.; Lepre, C. *J. Biol. Chem.* **2007**, *282*, 9733.

(11) Underwood, K. W.; Parris, K. D.; Federico, E.; Mosyak, L.; Czerwinski, R. M.; Shane, T.; Taylor, M.; Svenson, K.; Liu, Y.; Hsiao, C. L.; Wolfrom, S.; Maguire, M.; Malakian, K.; Telliez, J. B.; Lin, L. L.; Kriz, R. W.; Seehra, J.; Somers, W. S.; Stahl, M. L. *Structure* **2003**, *11*, 627.

(12) White, A.; Pargellis, C. A.; Studts, J. M.; Werneburg, B. G.; Farmer, B. T., II *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 6353.

(13) Mourey, R. J.; Burnette, B. L.; Brustkern, S. J.; Daniels, J. S.; Hirsch, J. L.; Hood, W. F.; Meyers, M. J.; Mnich, S. J.; Pierce, B. S.;

Saabye, M. J.; Schindler, J. F.; South, S. A.; Webb, E. G.; Zhang, J.; Anderson, D. R. *J. Pharmacol. Exp. Ther.* **2010**, *333*, 797.
(14) Llopis, A.; Salvador, N.; Ercilla, A.; Guaita-Esteruelas, S.; Barrantes Idel, B.; Gupta, J.; Gaestel, M.; Davis, R. J.; Nebreda, A. R.; Agell, N. *Cell Cycle* **2012**, *11*, 3627.
(15) Ehlting, C.; Ronkina, N.; Bohmer, O.; Albrecht, U.; Bode, K. A.; Lang, K. S.; Kotlyarov, A.; Radzioch, D.; Gaestel, M.; Haussinger, D.; Bode, J. G. *J. Biol. Chem.* **2011**, *286*, 24113.
(16) Steichen, J. M.; Iyer, G. H.; Li, S.; Saldanha, S. A.; Deal, M. S.; Woods, V. L., Jr.; Taylor, S. S. *J. Biol. Chem.* **2010**, *285*, 3825.
(17) Green, K. D.; Pflum, M. K. *J. Am. Chem. Soc.* **2007**, *129*, 10.
(18) Hertz, N. T.; Wang, B. T.; Allen, J. J.; Zhang, C.; Dar, A. C.; Burlingame, A. L.; Shokat, K. M. *Curr. Protoc. Chem. Biol.* **2010**, *2*, 15.
(19) Rowland, M. M.; Bostic, H. E.; Gong, D.; Speers, A. E.; Lucas, N.; Cho, W.; Cravatt, B. F.; Best, M. D. *Biochemistry* **2011**, *50*, 11143.
(20) Okerberg, E. S.; Wu, J.; Zhang, B.; Samii, B.; Blackford, K.; Winn, D. T.; Shreder, K. R.; Burbaum, J. J.; Patricelli, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4996.